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Epitope Mapping with Random Phage Display Library

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Abstract

Random phage display library is used to map conformational as well as linear epitopes. These libraries are available in varying lengths and with circularization. We provide here a protocol conveying our experience using a commercially available peptide phage display library, which in our hands provides good results.

Keywords

Allergen; Conformational epitope; Monoclonal antibody; Random phage display library

1 Introduction

It is believed that B cell epitopes, for instance aeroallergens, are generally conformational. Conformational epitopes are formed when several regions on the surface of proteins that are separated on the unfolded primary amino acid strands come together in the process of protein folding. Using random phage display libraries one can identify these epitopes, which are often not identified in overlapping peptide array experiments [1].

2 Materials

1. Random phage display library: Ph.D. Phage Display Libraries (New England Biolabs, NEB). Random peptides of 7 and 12 or circularized with cysteine residues are available from NEB. Additional protocols for these libraries are available from NEB.
2. Protein G magnetic beads (NEB), if monoclonal antibody (mAb) of interest is not mouse IgG, biotinylate it and use streptavidin magnetic beads.
3. Specific mAbs.
4. Isotype control antibody for preincubation of phage.
5. ER2738 *E. coli* strain (NEB).
6. LB medium.
7. SOC medium.

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8. IPTG/X-gal stock: Mix 1.25 g isopropyl-β-D-galactoside (IPTG) and 1 g 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) in 25 mL dimethyl formamide (DMF), Store at −0 °C.
9. LB/X-gal plates.
10. LB/IPTG/X-gal plates.
11. Top agar: Mix 10 g Bacto-Trypone, 5 g yeast extract, 5 g NaCl, 7 g Bacto-Agar, and MilliQ water to make up 1 L. Autoclave, and aliquot in 50 mL. Store at room temperature.
12. Tetracycline stock: 20 mg/mL in 1:1 ethanol:water. Store at −20 °C. Vortex before use.
13. LB/Tet plates.
14. Tris-buffered saline (TBS), pH 7.5: Add and mix NaCl (8.0 g), KCl (0.2 g), Tris base (6.1 g), and 800 mL of MilliQ water. Adjust pH to 7.5 with HCl. Make up to 1 L with MilliQ water. Autoclave, and store at room temperature.
15. Polyethylene glycol-8000 (PEG-8000)/NaCl: 20 % (w/v) PEG-8000, 2.5 M NaCl, autoclave, mix well to combine separated layers while still warm. Store at room temperature.
16. Iodide buffer: 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 4 M sodium iodide (NaI). Store at room temperature in the dark.
17. Streptavidin stock solution: 1.5 mg lyophilized streptavidin (supplied in NEB kit) in 1 mL 10 mM sodium phosphate, pH 7.2, 100 mM NaCl, 0.02 % NaN3. Store at 4 °C or −0 °C. Avoid freezing and thawing.
18. TTBS: 0.01 % TBS–Tween20 (50 mM Tris–HCl, pH 7.5, 150 mM NaCl).
19. Blocking buffer: 0.1 M $NaHCO_3$, pH 8.6, 5 mg/mL bovine serum albumin (BSA), 0.02 % NaN_3, 0.2 μm filter and store at 4 °C.
20. Sequencing primer (NEB).
21. Peroxidase labeled mouse anti-M13 phage antibody (Amersham).
22. 3,3,5,5-Tetramethylbenzidine (TMB) substrate (KPL, Pierce or Sigma).
23. Aerosol-resistant pipette.
24. Microcentrifuge tubes.
25. Toothpicks or gel-loading pipette tips, autoclaved.

3 Methods

3.1 Pre-cleaning Phage (See Note 1)

1. Take 50 μL of protein G or the appropriate beads in a 1.5 mL microcentrifuge tube.

2. Add 1 mL of TTBS. Suspend resin by tapping the tube.
3. Centrifuge tube for 30 s or use magnetic stand to pellet resin.
4. Remove supernatant.
5. Suspend resin in 1 mL blocking buffer. Incubate for 1 h at 4 °C with gentle rocking.
6. Pellet resin and discard supernatant.
7. Wash resin with 1 mL TTBS. Wash will be done with adding 1 mL TTBS to the tube, suspending resin by tapping the tube, centrifuging the tube for 30 s or using magnetic stand to pellet resin, and removing supernatant. Wash four times.
8. Dilute 1.5×10^{11} phage and 300 ng of isotype antibody to a final volume of 0.2 mL with TTBS. Final concentration of antibody is 10 nM.
9. Incubate at room temperature for 20 min.
10. Transfer the phage antibody mixture to the tube containing washed resin (Subheading 3.1, **step 7**).
11. Mix gently and incubate at room temperature for 15 min, mixing occasionally.
12. Wash ten times with 1 mL TTBS.
13. Elute bound phage by suspending pelleted resin in 1 mL 0.2 M glycine–HCl, pH 2.2, and in 1 mg/mL BSA. Incubate at room temperature for 10 min.
14. Centrifuge elution mixture for 1 min.
15. Carefully transfer the supernatant to a new microcentrifuge tube. Do not disturb the resin.
16. Immediately neutralize the eluate with 0.15 mL 1 M Tris–HCl, pH 9.1.
17. Titer the eluate.

3.2 Titering the Phage

1. Incubate 5 mL of LB with ER2738 with shaking for 4–8 h to OD 600 ~0.5.
2. Heat top agar in microwave and dispense 3 mL into sterile culture tubes, 1 tube/expected phage dilution. Maintain tubes at 45 °C.
3. Pre-warm LB/IPTG/X-gal plate/expected dilution for at least 1 h at 37 °C until ready to use.
4. Dilute serially to produce 10–10^3-fold dilution of phage in LB. Use the 10–10^4 dilutions for unamplified panning eluates and the 10^8–10^{11} dilutions for amplified culture supernatants.
5. When the culture reaches to OD_{600} ~0.5, dispense 0.2 mL into microcentrifuge tubes, 1 for each phage dilution.

[1]This is for removing the nonspecific phage binding to antibody using isotype antibody.

6. Add 10 μL of each phage dilution to individual tube to infect, vortex quickly, and incubate at room temperature for 1–5 min.
7. Transfer the infected cells, one infection at a time, to culture tubes containing 45 °C top agar.
8. Vortex briefly, and immediately pour culture onto a pre- warmed LB/IPTG/X-gal plate.
9. Gently tilt and rotate plate to spread top agar evenly.
10. Allow the plates to cool for 5 min.
11. Invert and incubate overnight at 37 °C.
12. Count plaques on those plates with about 100 plaques/plate.
13. Multiply each number by dilution factor to get the phage titer in plaque-forming unit (pfu).

3.3 Panning

1. Take 50 μL of protein G or other appropriately labeled beads in a 1.5 mL microcentrifuge tube.
2. Add 1 mL of TTBS. Suspend resin by tapping the tube.
3. Centrifuge tube for 30 s or use magnetic stand to pellet resin.
4. Remove supernatant.
5. Suspend resin in 1 mL of blocking buffer. Incubate for 1 h at 4 °C with gentle rocking.
6. Pellet resin, and discard supernatant.
7. Wash resin with 1 mL TTBS four times.
8. Dilute 1.5×10^{11} pre-cleaned phages (eluate phages from Subheading 3.1, **step 17**) and 300 ng of antibody of interest to a final volume of 0.2 mL with TTBS. Final concentration of antibody is 10 nM.
9. Incubate at room temperature for 20 min.
10. Transfer the phage/antibody mixture to the tube containing washed resin.
11. Mix gently, and incubate at room temperature for 15 min, mixing occasionally.
12. Wash ten times with 1 mL TTBS.
13. Elute bound phage by suspending pelleted resin in 1 mL 0.2 M glycine–HCl, pH 2.2, and 1 mg/mL BSA.
14. Incubate at room temperature for 10 min.
15. Centrifuge elution mixture for 1 min.

16. Carefully transfer the supernatant to a new microcentrifuge tube. Do not disturb the resin.
17. Immediately neutralize the eluate with 0.15 mL 1 M Tris–HCl, pH 9.1.
18. Titer the eluate (*see* Subheading 3.2).
19. Panning will be repeated three times (procedure Subheadings 3.3 and 3.4).

3.4 Amplification for Next Panning

1. Incubate 20 mL of LB with ER2738 with shaking for 4–8 h to OD 600 ~0.5.
2. Add the remaining phage from Subheading 3.3, **step 17**.
3. Incubate at 37 °C for 4.5–5 h with shaking.
4. Transfer the culture to a centrifuge tube and centrifuge for 10 min at 12,000 × *g* at 4 °C.
5. Transfer the supernatant to a new centrifuge and centrifuge for 10 min at 12,000 × *g* at 4 °C.
6. Transfer the upper 80 % to the new centrifuge tube, and add 1/6 volume of 20 % PEG/2.5 M NaCl.
7. Incubate at 4 °C for 2 h to overnight.
8. Centrifuge PEG precipitation at 12,000 × *g* for 15 min at 4 °C. Discard the supernatant. Double centrifuge for a short time, and remove supernatant with pipette.
9. Resuspend the pellet in 1 mL TBS by pipetting.
10. Transfer the suspension to a microcentrifuge tube and centrifuge at 14,000 rpm for 5 min at 4 °C.
11. Transfer the supernatant to a new microcentrifuge tube and reprecipitate with 1/6 volume of 20 % PEG/2.5 M NaCl.
12. Incubate for 15–60 min on ice.
13. Centrifuge at 14,000 rpm for 10 min at 4 °C. Double centrifuge for a short time, and remove supernatant with pipette.
14. Resuspend the pellet in 200 μL of TBS.
15. Centrifuge at 14,000 rpm for 1 min.
16. Transfer the supernatant to a new microcentrifuge tube. Amplified eluate can be stored for several weeks at 4 °C or stored at −0 °C by adding one volume of sterile glycerol.
17. Titer the amplified eluate on LB/IPTG/X-gal plate (Subheading 3.2).
18. Perform the next panning (Subheading 3.3).

3.5 Amplification and Purification of the Selected Phages

1. Culture ER2738 in 5 mL of LB with tetracycline (20 μg/mL) overnight.
2. Dilute overnight culture of ER2738 1:100 in LB.
3. Aliquot diluted culture in 14 mL tubes.
4. Select about 20–40 individual plaques from the plates after third panning (Subheading 3.2, **step 9**, *see* Note 2).
5. Stab a blue plaque by toothpick or gel-loading pipette tip and add to LB with ER2738 tube.
6. Incubate the tubes at 37 °C for 4.5–5 h with shaking.
7. Transfer 1.5 mL from the cultures into microcentrifuge tubes.
8. Centrifuge at 14,000 rpm for 3 min.
9. Transfer the supernatant to a new microcentrifuge tube and centrifuge again at 14,000 rpm for 3 min.
10. Transfer the upper 80 % of supernatant to a new microcentrifuge tube.
11. Transfer 500 μL of supernatant to a new microcentrifuge tube.
12. Add 200 μL of 20 % PEG-8000/2.5 M NaCl.
13. Mix gently by inverting tubes several times.
14. Let the microcentrifuge tube stand for 10–20 min at room temperature.
15. Centrifuge at 10,000 rpm for 20 min.
16. Discard supernatant.
17. Centrifuge quickly, and remove the remaining supernatant by pipetting.
18. Resuspend pellet in 1 mL TBS. 19. Store at 4 °C.

3.6 Phage ssDNA Extraction

1. Select amplified phage (1 mL) from individual clone to a new microcentrifuge tube.
2. Add 1/6 volume of 20 % PEG/2.5 M NaCl.
3. Incubate at room temperature for 10 min.
4. Centrifuge at 10,000 rpm for 10 min.
5. Discard the supernatant.
6. Suspend phage pellet thoroughly in 100 μL of iodide buffer followed by 250 μL 100 % ethanol.

[2]Plates should be <3 days old, stored in 4 °C, and should have <100 plaques.

7. Incubate the mixture for 10 min at room temperature to precipitate the single-stranded DNA.
8. Centrifuge at 10,000 rpm for 10 min.
9. Discard supernatant.
10. Add 0.5 mL of 70 % ethanol.
11. Centrifuge at 10,000 rpm for 10 min.
12. Discard supernatant.
13. Dry the pellet.
14. Suspend pellet in 30 μL of TE buffer (10 mM Tris–HCl [pH 8.0], 1 mM EDTA).
15. Quantify the product (5 μL) by agarose gel electrophoresis.

3.7 Nucleotide Sequencing

1. Sequence purified phage DNA with −6 g III primer 5′-HOCCC TCA TAG TTA GCG TAA CG -3′ (NEB). For instance while mapping for the major cockroach allergens Bla g 2 epitope, we sequenced 40 clones and obtained 32 clean sequences. Twenty-four out of 32 were SMMKADFDEEPR, and other 8 were SMMKADFEEEPR [3] (*see* Note 3).

   The small number of sequences recovered in this case suggested that the original library had limited phage sequences that interacted with the antibody 7C11 and that these were cleanly separated from relatively large number of other phage by this technique.

3.8 Phage ELISA (See Note 4)

1. Coat the plate with the antibody of interest (10 μg/ml) according to the plate manufacturer.
2. Add three dilutions (1:1, 1:10, 1:100) of phage diluted in TTBS to the wells. A nonspecific streptavidin-binding phage clone can be used as a negative control.
3. Incubate at 4 °C overnight.
4. Wash microtiter plate three times with TTBS.
5. Add peroxidase-labeled mouse anti-M13 phage antibody (Amersham).
6. Incubate at room temperature for 1 h.
7. Wash microtiter plate with TTBS four times.
8. Add TMB substrate.
9. Measure absorbance at 492 nm.

[3]To map the epitope on the molecule structure, there are several computational programs available. EpiSeach (http://curie.utmb.edu/episearch.html) has been recently modified, and we successfully used it in combination with crystal structure analysis [2, 3].
[4]After three rounds of selection, enrichment and specificity of the phage clones are confirmed by ELISA.

In our experiments, we tested for Bla g 2 epitope with 1×10^8–1×10^{15} PFU/mL. Significant inhibition of selected phages was observed in a dose–response manner [3].

3.9 Inhibition ELISA with Antibody-Specific Phage Clones (See [Note 5])

1. Coat microtiter plate with antibody (10 μg/ml in 100 μL borate buffer).
2. Incubate at 4 °C overnight.
3. Wash microtiter plate with TTBS three times.
4. Add phage clones at concentrations ranging from 10^7 pfu/mL to 10^{14} pfu/ml diluted in TTBS.
5. Incubate at 4 °C overnight. Unlabelled antigen is used as a negative control, and a nonspecific streptavidin-specific phage is used as a positive control.
6. Wash microtiter plate with TTBS three times.
7. Add biotin-labeled allergen at a concentration of 20 ng/mL.
8. Incubate microtiter plate ice for 30 min.
9. Wash microtiter plate with TTBS four times.
10. Add peroxidase-conjugated streptavidin diluted 1:1,000 in TTBS.
11. Incubate microtiter plate at room temperature for 1 h.
12. Wash microtiter plate with TTBS six times.
13. Add TMB substrate.
14. Measure absorbance at 492 nm.

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[5]Inhibition ELISA is performed to determine the degree of inhibition of allergen binding to antibody by the phage clones.